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transmembrane voltage. A further regulatory mechanism is channel inactivation, which occurs at the K+ selectivity filter and is of particular importance in the cardiac K+ channel hERG, that plays a major role in controlling the human heart rate. In a series of molecular dynamics simulations, we identified an extensive hydrogen bond network around residues S620 and N629 that controls the conformation of the selectivity filter and should therefore have a great impact on inactivation. We show that the distance of N629 to S620 acts as a nearly linear switch to drive the K+ selectivity filter from its conductive to a collapsed state similar to the low K+ conformation of KcsA. Electrophysiological measurements on mutants with altered H-bonding networks display either reduced or even fully abolished inactivation and thus support our hypothesis. Additionally, our simulations revealed a sidepocket extending from the central cavity, only present in the conductive state. These pockets are sufficently large to accommodate molecules of the newly found class of hERG-activators and reveal a first hint at their putative molecular mechanism.

2698-Pos Board B468
Testing Models for the Slow Inactivated State using Unnatural Amino Acid Mutagenesis
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K+ channels undergo a gating process called slow inactivation in which the selectivity filter changes conformation from a conductive state to a nonconductive state. Models for the structure of the selectivity filter in the slow inactivated state have been proposed based on structural studies of the KcsA K+ channel at low K+ or in the open state. Here, we experimentally evaluate the models for the slow inactivated state by using unnatural amino acid mutagenesis to precisely modify the selectivity filter. We have used the KcsA channel and the voltage gated K+ channel KvAP in our investigations. We will present the semisynthetic approaches used and describe the consequences of the unnatural amino acid substitutions on slow inactivation in the KcsA and KvAP channels.

2699-Pos Board B469
Shab K Channel Slow Inactivation. A Mechanism that Departs from Both C and U-Type Inactivation Mechanisms
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Once open Shab K channels inactivate within seconds. Shab slow inactivation presents characteristics that depart from both C and U-type mechanisms. Thus: Inactivation is facilitated by both external K^+ and TEA, and the steady-state inactivation curve does not have a U-shape. Interestingly, the intracellular pore blocker quinidine inhibits open-state inactivation. In addition to inactivate from the open state Shab presents significant closed-state inactivation, which is also facilitated by extracellular K^+. The time course of recovery from inactivation is the same regardless if channels inactivate from either the open or closed sates, indicating that in both cases they reach the same inactivated sate. The results herein presented combined with previous observations regarding the stability of G_K in 0 K^+ solutions at depolarized holding potentials, suggest that during Shab slow inactivation there is no closing or narrowing of the extracellular segment of the pore, capable to impede K^+ movement. It seems instead that there is only a narrowing or rearrangement of the intracellular portion of the pore that extends to its central cavity and halts ion conduction.

2700-Pos Board B470
Reducing S3-S4 Linker Length in Shaker K^+ Channels Stabilizes the Relaxed State
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The voltage sensing domain (VSD) of voltage-gated channels contains four transmembrane segments (S1 through S4), where the S4 segment is the main voltage sensor. It has been shown that upon prolonged depolarization the VSD enters a third conformational state, the relaxed state, resulting in a dramatic slowing of gating current kinetics upon a repolarization. The length of the linker between the third and fourth transmembrane domains (the S3-S4 linker) is highly variable between different voltage-gated potassium channels. Here we investigated whether the S3-S4 linker length affects the relaxation transition. We generated mutant clones of the Shaker K^+ (ShK) IR-H4, W434F background (wild type) with S3-S4 linkers progressively shortened by, typically, three residues. We found that upon prolonged depolarization, the kinetics of repolarizing gating currents going from the relaxed state to the resting state slow down with a linear correlation with the length of the S3-S4 linker ($R^2>0.9$). In addition, the entry to the relaxed state for short linker constructs was generally faster than that for wild type. These results show that shortening the S3-S4 linker favors the relaxed conformation. In addition, we recorded gating currents from oocytes expressing two fragments of ShK, one from the N terminus through part of the S3-S4 linker, and the other comprised of the remainder of the S3-S4 linker to the C terminus. This ''split linker'' construct and our short linker constructs suggest that shorter S3-S4 linkers impose greater constraints on the voltage sensor, stabilizing the relaxed state. Supported by NIH-GM030376.

2701-Pos Board B471
Voltage-Clamped Supported Bilayer System to Record Ion Channel Activity
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Structure-function studies of voltage-dependent ion channels require controlled electrical stimuli to drive channel activity. Under voltage control, site-directed fluorescence spectroscopy is often used to probe structural rearrangements that underlie channel function. Many voltage-dependent ion channels, such as the KvAP K+ channel, must be purified from bacteria and reconstituted in a lipid membrane. The ensuing (conventional) experiment in a black lipid membrane has several problems: 1) too few channels can be measured to observe gating currents; 2) the bilayer stability is generally poor and inversely related to protein density; 3) fluorescence analysis is difficult due to channel diffusion. To circumvent these problems, we previously developed a system to voltage clamp a supported bilayer with simultaneous fluorescence imaging (Hyde et al, 2010 BPS Meeting). This system employed a 5 mm diameter supported bilayer grown atop a transparent electrode-coated coverslip. We could measure gating currents of small charged molecules and voltage-dependent responses from fluorescent membrane probes; however, we have since found that direct contact with the supporting electrode significantly hinders recordings of functional channels. We have thus introduced a self-assembled monolayer cushion grown atop a gold-coated coverslip as the support electrode. Importantly, the gold-coated coverslip is also designed to enable simultaneous fluorescence imaging of ion channels in the supported bilayer using surface plasmon-assisted microscopy. Preliminary results indicate that in response to an applied transmembrane potential, we can observe voltage-dependent fluorescence changes from S4-labeled KvAP channels consistent with presumptive gating behavior. Support: NIH GM030376; Medical Scientist National Research Service Award 5 T32 GM007281.

2702-Pos Board B472
Position of the Second Gating Charge along S4 in an Intermediate Conformation of a K^+ Channel Voltage Sensor
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Voltage-sensing in ion channels and some phosphatases relies on the movement of positively-charged residues (gating charges) carried by the fourth (S4) helix of the voltage-sensor domain (VSD). Structures of Kv channels revealed the position of the gating charges in the open/inactivated conformation of the VSD yet the pathway they follow during gating remains unclear. To this aim, we previously showed that at hyperpolarized potentials, the first gating charge of the Shaker K+ channel (R362) resides within atomic proximity of I241, located in the middle of the S1 segment. Here, we show that I241 mutations shift the voltage dependence of channels activation toward positive potentials and this effect is most pronounced for the I241W mutation. This mutation isolates two equal components of the charge movement during activation, thereby stabilizing the S4 in an intermediate position between resting and active states. Using non-natural amino acid incorporation, electrophysiology recordings and molecular dynamic simulations, we show that this S4-immobilization involves a specific interaction between I241W and R365, the second gating charge. We found that this interaction requires the presence of the nitrogen atom of the indole ring and is not affected by a dispersion of its Pi electrons. This interaction is present regardless of whether the S4 moves from its resting, active or relaxed state but the charge component that is being isolated during deactivation only represents 30% compared to 50% during activation. Taken together, our results indicate that each of the gating charges move in slightly different pathways, the overall S4 motion is similar during activation and deactivation, and the electric field is shaped differently between activation and deactivation.

2703-Pos Board B473
An S4-S5 Linker Residue Influences Kv2.1 Channel Gating and Inhibition by Carbon Monoxide Releasing Molecule 2 (CORM-2)
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The carbon releasing molecule 2 (CORM-2) is an allosteric inhibitor of the Shaker and Kv2.1 voltage-gated ion channels. The mechanism of inhibition is common to both channels and derives mainly from an energetic destabilization

of the channel's opening transition without inhibiting voltage-sensor activation, thus partially uncoupling voltage-sensor activation from channel gating. Electromechanical coupling between the voltage-sensing domain (VSD) and the pore of Kv channels has been shown to depend on specific interactions between the S4-S5 linker and the carboxyl-terminal portion of the S6 segment. Here, we show that a single glycine to valine mutation in the S4-S5 linker of the Kv2.1 channel causes a large energetic destabilization of the channel's open state. Moreover, the G317V mutation also interferes with the channel's inhibition by CORM-2, suggesting that there is a cavity at the interphase between the VSD and the pore of the channel, including the S4-S5 linker, and that it is part of the CORM-2 binding site in the channel. These data are consistent with the mechanism of channel inhibition by this compound. Supported by Instituto de Ciencia y Tecnología del Distrito Federal, Grant PIFUTP09-262 to L.D.I.

2704-Pos Board B474

Intracellular Ions Impede Voltage Sensor Return in Kv1.2 Channels

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Voltage sensing in Kv channels originates from the coupling of movement between the charged S4 segment and the activation gate at the cytoplasmic region of the pore domain. The voltage sensor moves prior to the pore opening and the pore must shut before the voltage sensor returns to its resting state. However, gating current recordings from Kv channels indicate that frequently voltage sensors return more slowly after depolarisations that populate open states, indicating that the open pore exerts a resistance to S4 return. This process of pore closure therefore intrinsically regulates the deactivation kinetics of Kv channels. We observed slow voltage sensor return (Ig_{OFF}) in WT-Kv1.2 channels under non-permeant ionic conditions after depolarisations to voltages that caused channel openings. Using TEA^+ and NMG^+ internal solutions resulted in a slower Ig_{OFF} than internal Cs^+, suggesting that the intracellular ionic composition was modulating Ig_{OFF}. A mutation in the pore lining S6 segment to enlarge the inner cavity (Kv1.2-I402C) removed the slowing of Ig_{OFF} in the presence of internal NMG^+, suggesting that NMG^+ interacted within the inner cavity of the WT channel to prevent pore closure through a 'foot in the door' mechanism. Gating currents of a non-conducting, P-type inactivated channel (Kv1.2-W366F, V381T), in the presence of intracellular K^+ ions also displayed a slowing of Ig_{OFF} after depolarisations that would open the channel pore. These results suggest that internal K^+ ions bound in the inner cavity can also slow activation gate closure. We propose that internal ions in the cavity of Kv1.2 allosterically regulate the voltage sensor deactivation kinetics by preventing pore closure and thus rate limiting the return of voltage sensors.

2705-Pos Board B475

Voltage-Dependent Gating of the K+ Channel KvLm Explored through Heterotetramers

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Voltage-gated K+ (Kv) channels are tetrameric assemblies in which each subunit is modular in design and consists of a voltage-sensor and a pore. KvLm, the voltage-gated K+ channel from Listeria monocytogenes differs from other Kv channels in that its voltage-sensor contains only three out of the eight charged residues implicated in voltage-gating. Here we ask how many sensors are required to produce a functional Kv channel by investigating heterotetramers comprising combinations of KvLm full-length (FL) and its sensorless pore-module (PM). Accordingly, we studied the voltage-dependent properties of KvLm channels with 0, 1, 2, 3 and 4 voltage sensors. We show that KvLm heterotetramers produced by cell-free expression yield functional channels after reconstitution in droplet interface bilayers. Further, we demonstrate that three voltage-sensors are sufficient to recapitulate the voltage-dependent activation features of wild-type KvLm, whereas deletion of two or more sensors severely suppresses the voltage-dependent closure and activation of the assembled channel. The current-voltage relationship of all heteromers remains similar. We also demonstrate that all four voltage-sensors are required to keep the channel closed at hyperpolarizing potentials, and that deletion of all four sensors results in a pore-only assembly, which retains limited voltage-dependence.

2706-Pos Board B476

Allosteric Stabilization of Fully Resting Voltage Sensors by a Tarantula Toxin

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The mechanism of a tarantula toxin's action on the voltage gating of a K channel was investigated. An oxidation-resistant variant of guangxitoxin-1E (GxTX) with methionine 35 replaced by norleucine, was synthesized and found to retain biological activity. When applied to voltage-clamped CHO-K1 cells expressing rat Kv2.1, this GxTX was found to shift channel opening to more positive voltages. In response to short stimulating voltage steps, the voltage shift of conductance saturated at micromolar GxTX concentrations. Prolonged or repetitive pulses to positive potentials ejected GxTX from Kv2.1 channels, revealing the decreased affinity of GxTX for activated voltage sensors. GxTX positively shifted Kv2.1 gating currents, and prevented outward gating charge movement at negative voltages. The modulation of gating charge movement indicates that GxTX stabilizes gating charges in their most internal conformation. Single Kv2.1 channels with GxTX bound exhibited a similar unitary conductance as without tarantula toxin, but had an increased latency to first opening in response to positive voltage steps. A diminished mean open time in the presence of GxTX confirms that channel openings occur with toxin bound and suggests a mechanism for the toxin-induced decrease in peak conductance of macroscopic currents. A simple allosteric model was developed where GxTX stabilizes the earliest resting state of voltage sensors. In this model, GxTX binds activated voltage sensors with decreased affinity, and exerts only a feeble destabilizing influence on the dominant open state. The difference in binding affinity between resting and activated voltage sensors suggests potential for development of GxTX as a probe of voltage sensor conformation in living cells.

2707-Pos Board B477

Activation Gate Opening Precedes Slow Inactivation in Shaker K+ Channels

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In the absence of N-type inactivation Shaker potassium channels display slow (C-type) inactivation. Functional and structural studies indicated that channel opening precedes slow inactivation in voltage-gated and KcsA K+ channels, whereas others argued for slow inactivation from the closed sate of the channel as well. None of the previous studies correlated the opening of the of the activation gate (A-gate), formed by the bundle crossing of the S6 segments, with the development of inactivation, which is associated with the structural rearrangement in the selectivity filter. These two gates are coupled and thus, the current experiments addressed the hypothesis that opening of the activation gate must precede slow inactivation.

To address this hypothesis we compared the voltage dependence of A-gate opening and that of the development of inactivation in T449A/V474C Shaker-IR channels. Opening of the A-gate was monitored by the accessibility of 474C to Cd2+ from the intracellular side. The membrane potential was changed repeatedly from a holding potential of −120 mV to test potentials ranging from −110 mV to −60 mV in the presence or absence of Cd2+.

Our results show that the function describing the voltage dependence of Cd2+ block is shifted toward the negative potentials compared to the voltage dependence of steady-state inactivation curve. This indicates that A-gate opening already occurs at such negative potentials where no inactivation can be detected. Furthermore, the curve representing Cd2+ block is also negative-shifted compared to the voltage dependence of steady-state activation (G-V) curve. This suggests that even at fairly negative holding potentials, at which no macroscopic current can be detected, rare channel openings occur yielding access to the channel cavity. Based on these results we suggest that A-gate opening always precedes structural changes associated with slow inactivation.

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2708-Pos Board B478

Gating Properties and Voltage Sensing in Kv1.2

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Kv1.2 is a voltage gated potassium channel whose crystal structure has been solved. There is markedly little electrophysiological information about it, which limits the vast potential usefulness of the structure. In an effort to deepen our functional understanding of the Kv1.2 channel, we set out to characterize its properties through recordings of macroscopic and single ionic and gating currents and develop models for activation based on established models for other Shaker channels. Preliminary data indicate that even though Shaker and Kv1.2 are closely related, subtle differences exist in their mechanisms of voltage sensing. As an example of these differences, limiting slope analysis reveals that the apparent charge coupled to gating of Kv1.2 is only 10 e0 compared to Shaker's 13 e0. Also, substitution of Kv1.2's tryptophan at position 366, the analogue of W434 in Shaker, for the other two aromatic residues does not abolish conduction as in Shaker. Our macroscopic data are best fitted by a 32 state model involving three independent transitions for each of the subunits and two final, voltage independent, concerted transitions to the open state.